The Invention

The present invention provides a two-component system to produce a lethal effect in plant cells. In this system, two polypeptides are expressed in a plant cell. The polypeptides are encoded by expression cassettes located at the same locus on each of two homologous chromosomes. One expression cassette comprises a first promoter operably linked to a first polynucleotide sequence, with a recombinase site between the first promoter and the first polynucleotide sequence. The second expression cassette comprises the first plant promoter inoperably linked to the first polynucleotide due to the presence of an intervening expression cassette, flanked by two recombinase sites, between the first promoter and the first polynucleotide. The intervening expression cassette comprises a second promoter operably linked to a second polynucleotide.

Each expression cassette of the invention is individually functional, but the product of each cassette alone does not provide the desired lethal effect. The combination of the two polypeptides from the individual expression cassettes is required for producing the lethal effect. The first and second polypeptides can either be separate functional polypeptides from distinct loci, or nonfunctional polypeptide subsequences that together produce a single functional polypeptide.

Rejection under 35 U.S.C. § 112, first paragraph: enablement

Claims 1-4, 6, 7, 11-18, 20, 21, and 25-37 were rejected as allegedly lacking enablement. The rejection states that Applicants have not disclosed transgenic plants that are transformed with the expression constructs as claimed, and therefore, the claimed methods are unpredictable. Office Action, page 3, lines 13-15. The rejection also states that the use of inducible promoters is unpredictable. *Id.*, page 3, line 18 to page 4, line 4 . The rejection further states that it is unpredictable whether transgenes will be expressed as expected once they are integrated into the genome. *Id.*, page 4, lines 4-9. Effectively, the rejection appears to argue that one of skill in the art would not be able to create transgenic plants, using an expression vector encoding a nuclease that is lethal to the cell, without undue experimentation.

PATENT

The proper test of enablement is "whether one skilled in the art could make or use the claimed invention from the disclosure in the patent coupled with information known in the art without undue experimentation." *See, e.g.*, MPEP § 2164.01. As identified by the Patent Office and the Federal Circuit, whether undue experimentation is required by one skilled in the art to practice the invention is determined by considering factors such as the breadth of the claims, the level of one of ordinary skill, amount of guidance presented in the application, and the presence of working examples. *Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int. 1985); *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). As described in *Wands*, "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should precede." *Wands*, 8 USPQ2d at 1404 (quoting *In re Jackson*, 217 USPQ 804 (Bd. Pat. App. & Int. 1982).

Applicants first note that they have previously amended the claims to recite that at least one of the expression cassettes encodes an amino acid sequence from a nuclease, whose expression produces the desired lethal effect in particular cells. However, to the extent that the rejection remains applicable to the claims as amended, Applicants respectfully traverse. The specification provides guidance for making expression vectors that express the nucleases of the invention, and selecting promoters for expression of the nucleases in plants. Furthermore, methods of transforming plant cells are well known in the art, and Applicants present evidence that the claimed methods can be used to make transgenic plants wherein the desired lethal effect is produced in cells, using either a constitutive or a tissue-specific promoter. Finally, one of skill in the art would be able routinely to screen and identify transgenes that are expressed in plant cells. One of skill in the art would therefore be able to practice the claimed invention with, at most, only routine experimentation.

A. The specification describes combinations of promoters and expression cassettes for use in the invention

Applicants submit that it would be routine for one of skill in the art to make expression vectors expressing nuclease polypeptides or polypeptide fragments in selected cells. As described in the specification, many nucleic acid sequences are known for members of the

deoxyribonuclease and ribonuclease families, and new sequences from these conserved families could be easily identified using routine methodology (*see, e.g.*, specification at page 16). Techniques for recombinant production of polypeptide are well known to those of skill in the art. Furthermore, the nucleases of the invent can easily be tested for activity using any one of a number of well known assays to test nuclease function (*see, e.g.*, specification page 17). As described in the declaration of Dr. Neal Gutterson, submitted herewith, expression vectors expressing 5' and 3' barnase polypeptide subsequences, when expressed together in a transgenic plant, produce the desired lethal effect.

The present specification also fully describes the use of a number of different promoters, including constitutive and non-constitutive promoters such as inducible and tissue-specific promoters, to achieve this result (*see, e.g.*, specification, page 9, line 22 to page 12, line 18). For example, the specification describes that one may use a combination of promoters whose expression overlaps, e.g., a constitutive promoter and a tissue-specific promoter, or a combination of two tissue-specific promoters, or a combination of inducible promoters. In addition, as described in the declaration of Dr. Neal Gutterson, submitted herewith, expression vectors expressing 5' and 3' barnase polypeptide subsequences, when expressed together in a transgenic plant using either a constitutive 35S promoter, or a tissue specific tapetal promoter, produce the desired lethal effect in the selected cells.

Although the rejection expressed concern about "leaky promoters," this situation is not an issue for the present invention, although it may be applicable to the one component systems of the prior art. In the two component system of the invention, expression of both components in the same cell is required for the lethal effect. Therefore, all that is required is that expression from the selected promoters overlaps in the desired cell type.

B. Making transgenic plants of the invention is predictable, as described in the specification and demonstrated by transgenic plant data presented herewith

In the field of plant biotechnology, transformation of a variety plant species and expression of a recombinant gene of choice from a plant promoter is merely routine. In addition, the specification provides guidance for transforming plant species. Finally, Applicants present a declaration of Dr. Neal Gutterson, providing experimental results of

transgenic plants transformed with expression vectors that impair cellular function in selected plant cells. The assays of the specification, together with experimental data provided herewith and the standard methodology known to those of skill in the art, therefore provide adequate guidance for using the claimed methods to express a recombinant <u>nuclease</u> nucleic acid in a wide variety of plants.

<u>1. The specification and known methodology teach how to make and use the methods of the invention</u>

At the time of the present invention, transformation of plant species with a variety of expression vectors encoding a particular recombinant gene, such as a nuclease, was well within the means of one of skill in the art, without undue experimentation. For example, suitable techniques for plant transformation and regeneration are described, e.g., Klee, *Ann. Rev. of Plant Phys*, 38:467-486 (1987); Weising *et al., Ann. Rev. Genet.* 22:421-477 (1988); *Handbook of Plant Cell Culture* (1983); and Binding, *Regeneration of Plants* (1985). Such techniques are standard methodology and are well known to those of skill in the art.

The present application also provides considerable direction and guidance for practicing the claimed invention, as the specification describes methods of transforming plant cells and regenerating plants. For example, the specification describes how to perform both transient and stable transformation methods. Numerous different methods for plant transformation are described, including methods such as electroporation, microinjection, ballistic methods, *Agrobacterium* transformation, and PEG precipitation (*see, e.g.*, specification, page 8, line 23 to page 9, line 21). In addition, the specification also describes regeneration techniques for generating plants from transformed plant cells (*see, e.g.*, specification, page 19, lines 1-31). Applicants thus submit that transformation of a plant with expression cassettes encoding a nuclease requires, at most, only routine experimentation.

<u>2. Transgenic plant experiments demonstrate that the claimed methods can be routinely practiced by one of skill in the art</u>

As described in the declaration under 37 C.F.R. § 1.132 of Dr. Neal Gutterson, submitted herewith, plant experiments have been performed in which two separate

subsequences of a single functional protein (barnase, a ribonuclease) were expressed in transgenic plants. Dr. Gutterson supervised these experiments. In one experiment, the barnase polypeptides were expressed in transgenic tomato plants using a constitutive promoter. In another experiment, the barnase polypeptides were expressed in transgenic tomato plants using a tapetal-specific promoter. In both experiments, the barnase polypeptide subsequences formed a functional barnase protein that impaired cellular function in the cells in which the polypeptide subsequences were expressed.

In one experiment, expression of 5' and 3' barnase polypeptide subsequences in the same cells, using a constitutive promoter, was lethal to the plant. Expression vectors were made that encoded either a 5' barnase polypeptide (p35S/Bn5-2) or a 3' barnase polypeptide (p35S/Bn3-2), each operably linked to the Cauliflower mosaic virus 35S promoter (a constitutive promoter). Transgenic tomato plants were transformed with either the 5' barnase expression vector or the 3' barnase expression vector, according to standard techniques, and these plants expressed their respective integrated transgenic constructs.

To determine whether expression of Bn5-2 and Bn3-2 together using a constitutive promoter would be lethal to the plant, transgenic tomato plants expressing either p35S/Bn5-2 or p35S/Bn3-2 were crossed, and the resulting seeds were planted. In one cross, thirty nine seeds were planted. Both parents were hemizygous and had transgenes at two loci, so that approximately 55% of the progeny were expected to inherit both transgenes. Twenty (51%) of the seeds did not germinate. Among the 19 seeds that did germinate, none carried both transgenes. For all crosses combined, out of 87 seeds planted, only 35 of these seeds germinated and produced normal-looking tomato plants (*see* Table 1, Exhibit 2). None of these normal plants carried both the Bn5-2 and the Bn3-2 constructs. Besides the normal looking tomato plants, two seeds germinated but died after making only a hypocotyl and a few roots. PCR analysis detected both the Bn3-2 and the Bn5-2 constructs in those two plants. These results are consistent with the prediction that the combination of both transgenes would be lethal to the plant.

In another experiment, expression of the 5' and 3' barnase polypeptide subsequences together in specific cells (the tapetum), using a tissue specific promoter, resulted

in impairment of the cells and male sterility. As described above, transgenic tomato plants were transformed according to standard methodology with either a Bn5-2 or a Bn3-2 transgene construct operably linked to a p127a promoter, a tomato tapetal-specific promoter. Expression of both the Bn3-2 and the Bn5-2 integrated transgenes peaked at the 5-7 mm bud stage, which corresponds to the tetrad stage of microspore development when the tapetum is maximally active.

To determine whether expression of Bn5-2 and Bn3-2 together in the tapetum would be lethal to pollen generation, transgenic tomato plants expressing either p127a /Bn5-2 or p127a /Bn3-2 were crossed, and the resulting seeds were planted. In one cross, one of three seeds germinated. This plant inherited both Bn5-2 and Bn3-2 and was male sterile. The anthers on the plant were flat but not reduced in size. The plant had normal flower and plant morphology. It did not produce any selfed fruit, but was female fertile, producing fruit when outcrossed as a female. Seed has been successfully germinated from the outcrossed fruit. Additional experiments have demonstrated co-segregation of: (1) the two transgenes (Bn5-2 and Bn 3-2) and (2) the male sterile phenotype.

When a flower from the Bn5-2/Bn3-2 plant was examined, only 64 pollen-like grains were found. These grains differed morphologically from normal pollen grains, being less refractile, smaller, and shriveled in appearance. None of these pollen grains were viable, according to a fluorescein diacetate staining assay. In comparison, control flowers, including controls corresponding to both parents, had between 2400 to 7300 pollen grains per flower, with 39-51% of the pollen grains being viable according to the fluorescein diacetate staining assay. These results are consistent with the prediction that the combination of both transgenes would be lethal to pollen generation.

Applicants thus submit that transformation of a plant with expression cassettes encoding a nuclease requires, at most, only routine experimentation.

C. Screening and identification of expressed transgenes is routine to one of skill in the art

The rejection also appears to argue that plant transformants may be inoperable due to the unpredictability of transgene expression. However, claims reading on inoperative

embodiments are enabled if the skilled artisan understands how to avoid the inoperative embodiments. As described by the Court of Customs and Appeals:

> [M]any patented claims read on vast numbers of inoperative embodiments in the trivial sense that they can and do omit 'factors which must be presumed to be within the level of ordinary skill in the art.' . . . There is nothing wrong with this so long as it would be obvious to one of skill in the art how to include these factors in such manner as to make the embodiment operative rather than inoperative. *In re Cook and Merigold*, 169 USPQ 299, 302 (C.C.P.A. 1971) (quoting *In re Skrivan*, 166 USPQ 85, 88 (C.C.P.A. 1970)).

Thus, even if we assume, *arguendo*, that activation of a transgene fails to occur in some instances of plant transformation, this situation does not indicate that the claims lack enablement. One of skill in the art would clearly understand that another transgenic plant could be routinely created in its place. As previously described, transformation of plants with recombinant genes is now considered routine, as demonstrated, e.g., by the following laboratory manuals: *Gene Transfer to Plants* (Potrykus & Spangenberg, eds., 1995); and *Methods in Molecular Biology 49: Plant Gene Transfer and Expression Protocols* (Jones & Walkers, eds., 1995). One of skill in the art would therefore recognize how to overcome any difficulties arising from expression of a transgene in a transgenic plant.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested. If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

Annette S Parent

Annette S. Parent
Reg. No. 42,058

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: 415-576-0200
Fax: 415-576-0300
SF 1181304 v1

APPENDIX--PENDING CLAIMS

1. (previously twice amended) A plant containing a plant cell comprising a first and a second expression cassette located at the same locus on each of two homologous chromosomes, wherein:

the first expression cassette present on a first chromosome homolog comprises a first plant promoter operably linked to a first polynucleotide sequence encoding a first polypeptide, wherein a recombinase site is present between the first promoter and the first polynucleotide sequence;

the second expression cassette present on a second chromosome homolog comprises the first plant promoter inoperably linked to the first polynucleotide sequence, wherein an intervening expression cassette is flanked by two recombinase sites and situated between the first promoter and the first polynucleotide sequence of the second expression cassette, the intervening expression cassette comprising a second plant promoter operably linked to a second polynucleotide sequence encoding a second polypeptide;

wherein at least the first or the second plant promoter is a non-constitutive promoter; wherein at least the first or the second polynucleotide encodes an amino acid sequence of a nuclease or wherein the first and second polynucleotides each encode a separate amino acid subsequence of a single functional nuclease; and

wherein the presence of the first and second polypeptides in a cell is lethal to the cell.

2. (as filed) The plant of claim 1, wherein the recombinase sites are lox sites.

3. (as filed) The plant of claim 1, wherein the first polypeptide is a transactivator protein.

4. (as filed) The plant of claim 1, wherein the intervening expression cassette is in reverse orientation with respect to the second expression cassette.

6. (previously twice amended) The plant of claim 1, wherein at least the first or the second polynucleotide encodes an amino acid sequence of a ribonuclease or wherein the first and second polynucleotides each encode a separate amino acid subsequence of a single functional ribonuclease.

7. (as filed) The plant of claim 6, wherein the ribonuclease is Barnase.

11. (as filed) The plant of claim 1, wherein the first or the second promoter is a tissue-specific promoter.

12. (as filed) The plant of claim 1, wherein the first and second promoters are each functional in tapetal cells.

13. (previously once amended) The plant of claim 1, wherein the first and second polypeptides each comprise a separate subsequence of a single functional nuclease polypeptide.

14. (previously twice amended) A method of modifying cellular function in a plant, the method comprising the steps of:

introducing into a plant a first expression cassette comprising a first plant promoter operably linked to a first polynucleotide encoding a first polypeptide, wherein a recombinase site is present between the first promoter and the first polynucleotide;

introducing into the plant a second expression cassette comprising the first plant promoter inoperably linked to a polynucleotide encoding the first polypeptide, wherein an intervening expression cassette is flanked by recombinase sites and situated between the first promoter and the first polypeptide of the second expression cassette, the intervening expression cassette comprising a plant promoter operably linked to a polynucleotide encoding a second polypeptide;

wherein at least the first or the second plant promoter is a non-constitutive promoter; wherein at least the first or the second polynucleotide encodes an amino acid

sequence of a nuclease or wherein the first and second polynucleotides each encode a separate amino acid subsequence of a single functional nuclease; and

wherein the presence of the first and second polypeptides in a cell is lethal to the cell.

15. (as filed) The method of claim 14, wherein the two expression cassettes are introduced through a sexual cross and the two expression cassettes are present on chromosome homologs.

16. (as filed) The method of claim 14, wherein the recombinase sites are lox sites.

17. (as filed) The method of claim 14, wherein the first polypeptide is a transactivator protein.

18. (as filed) The method claim 14, wherein the intervening expression cassette is in reverse orientation with respect to the second expression cassette.

20. (previously twice amended) The method of claim 14, wherein at least the first or the second polynucleotide encodes an amino acid sequence of a ribonuclease or wherein the first and second polynucleotides each encode a separate amino acid subsequence of a single functional ribonuclease.

21. (as filed) The method of claim 20, wherein the ribonuclease is Barnase.

25. (as filed) The method of claim 14, wherein the first or the second promoter is a tissue-specific promoter.

26. (as filed) The method of claim 14, wherein the first and second promoters are each functional in tapetal cells.

27. (previously once amended) The method of claim 14, wherein the first and second polypeptides each comprise a separate subsequence of a single functional nuclease polypeptide.

28. (as filed) The plant of claim 1, wherein both the first and the second promoters are non-constitutive promoters.

29. (as filed) The plant of claim 1, wherein the first and second promoters have overlapping specificities.

30. (as filed) The plant of claim 1, wherein the first or the second promoter is a seed coat-specific promoter.

31. (as filed) The plant of claim 6, wherein the ribonuclease is ribonuclease T1 or binase.

32. (as filed) The plant of claim 6, wherein the first and second polypeptides each comprise a separate subsequence of a single functional ribonuclease polypeptide.

33. (as filed) The plant of claim 14, wherein both the first and the second promoters are non-constitutive promotes.

34. (as filed) The method of claim 14, wherein the first and second promoters have overlapping specificities.

35. (as filed) The method of claim 14, wherein the first or the second promoter is a seed coat-specific promoter.

36. (as filed) The method of claim 20, wherein the ribonuclease is ribonuclease T1 or binase.

37. (as filed) The method of claim 20, wherein the first and second polypeptides each comprise a separate subsequence of a single functional ribonuclease polypeptide.